

# A Subpopulation of Cardiomyocytes Expressing $\alpha$ -Skeletal Actin Is Identified by a Specific Polyclonal Antibody

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**Abstract**—The NH<sub>2</sub>-terminal decapeptide of  $\alpha$ -skeletal actin that contains a primary sequence specific for this isoform was used to raise a polyclonal antibody in rabbits. Using sequential affinity chromatography, we recovered from serum antibodies reacting exclusively with  $\alpha$ -skeletal actin when tested by immunoblotting and immunofluorescence. Epitope mapping by means of competition assays with synthetic peptides indicated that the acetyl group and the first 9 amino acids are essential for specificity. The monospecific antibody was then used to investigate the distribution of  $\alpha$ -skeletal actin in the myocardium of newborn and normal or hypertensive (with or without fibrotic areas) adult rats. Immunostaining of normal heart revealed that  $\alpha$ -skeletal actin is diffusely distributed within practically all myocardial fibers of the newborn rat, whereas it is restricted to a small proportion of adult rat cardiomyocytes, which appear intensely stained. A correlation, albeit not complete, was found between the distribution of  $\alpha$ -skeletal actin and  $\beta$ -myosin heavy chain. During cardiac hypertrophy induced by aortic ligation between the renal arteries, the expressions of  $\alpha$ -skeletal actin mRNA and protein were increased. The distribution of immunostaining had a focal pattern similar to that of normal adult rats, reactive fibers being more numerous and more intensely stained compared with normal myocardium. Positive fibers were particularly abundant at the periphery of fibrotic areas. Using this antibody, we have demonstrated for the first time the differential distribution of  $\alpha$ -skeletal actin in heart tissues. Changes in the distribution of this isoform in hypertrophic heart provide new insight into the mechanisms by which the heart adapts to work overload. This antibody will prove useful in exploring the mechanisms of expression of  $\alpha$ -skeletal actin and in defining its role in physiological and pathological situations. The full text of this article is available at <http://www.circresaha.org>. (*Circ Res.* 1999;85:e51–e58.)

**Key Words:** actin isoform ■ cardiac hypertrophy ■ fibrosis ■ myocardium ■ hypertension

The 6 actin isoforms found in mammals constitute a family of closely related proteins expressed in a tissue-specific way.  $\alpha$ -Skeletal and  $\alpha$ -cardiac actins are the preponderant actin isoforms detected in striated muscles.<sup>1</sup> Their sequences are almost identical, differing by only 4 out of 375 amino acids.<sup>2</sup> Two of these differences consist of an inversion of glutamic and aspartic acid at residues 2 and 3 in the NH<sub>2</sub>-terminus of the protein. The nucleic acid sequences of mRNA coding regions are similarly well conserved; however, these 2 isoactin mRNAs markedly differ in their 5' and 3' untranslated regions, which can be used to unambiguously distinguish between them. Numerous studies have taken advantage of these differences to show that the expression of each mRNA varies quantitatively with species, muscle type, development, or pathological situations.<sup>3–6</sup> It has been shown that the 2 sarcomeric mRNAs are coexpressed in skeletal and cardiac muscle:  $\alpha$ -cardiac actin mRNA has been found in developing mouse,<sup>7</sup> rat,<sup>8</sup> and chick<sup>9</sup> skeletal muscles. Conversely, the myocardium of various species contains the mRNA encoding skeletal actin, in proportions varying during development and/or pathological situations. In the rat heart, both isogenes are expressed in utero; the proportion of total

actin mRNA due to  $\alpha$ -skeletal actin mRNA is 28% at 17 to 19 days of fetal development, 40% one week after birth and 5% in the adult.<sup>5</sup> An increase in skeletal actin mRNA expression also has been demonstrated in a variety of experimental models of hypertension with consequent myocardial hypertrophy.<sup>3,10–12</sup> At the protein level, studies on actin isoform distribution and quantification have been carried out using electrophoresis and by analysis of the partially hydrolyzed NH<sub>2</sub>-terminal peptides.<sup>1,13</sup> Using these methods, it was shown that both  $\alpha$ -skeletal and  $\alpha$ -cardiac actins are simultaneously expressed in various types of striated muscle and that a good correlation between the relative protein and mRNA expressions generally is observed. However, no description of the distribution of these isoforms at the cellular level heretofore has been presented because of the lack of an antibody recognizing unambiguously one of them, despite the existence of antibodies specific for other actin isoforms.<sup>14–16</sup> This has left open questions regarding the distribution of  $\alpha$ -skeletal actin protein expression in muscle tissues, as well as the degree to which the 2  $\alpha$ -striated muscle actins colocalize in the same cell and within the sarcomere.

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Exhibit 7

In the present work, we report the production, immunopurification, and characterization of an antibody raised in the rabbit against a synthetic decapeptide corresponding to the NH<sub>2</sub>-terminal sequence of  $\alpha$ -skeletal actin. Using this antibody, we have established the localization of  $\alpha$ -skeletal actin in the normal rat myocardium during postnatal development and provided new insight regarding its distribution during the evolution of an experimental model of hypertension.

## Materials and Methods

### Peptide Synthesis

Decapeptides with the NH<sub>2</sub>-terminal sequences of the 6 mammalian actin isoforms were synthesized as previously described.<sup>17</sup> Shorter  $\alpha$ -skeletal actin peptides with 5, 7, 8, and 9 amino acids at the NH<sub>2</sub>-terminal extremity were synthesized by Research Genetics.

### Preparation and Purification of the Anti- $\alpha$ -Skeletal Actin Antibody (Anti- $\alpha$ -SKA1)

The NH<sub>2</sub>-terminal decapeptide of  $\alpha$ -skeletal actin (Ac-DEDETTALVC-COOH) was coupled to maleimide-activated key-hole limpet hemocyanin (KLH, Pierce) through its cysteine residue according to the instructions of the manufacturer. The coupled peptide was used for antiserum production in a rabbit (performed by Biotools International).

The antiserum was first affinity purified using Sulfolink beads (Pierce) coupled with the  $\alpha$ -skeletal decapeptide. Antibodies bound to the column were eluted with 0.2 mol/L glycine-HCl pH 2.8 and 0.5 mol/L NaCl, dialyzed overnight against phosphate-buffered saline solution (PBS), and then loaded on a Sulfolink column coupled with the NH<sub>2</sub>-terminal decapeptide of  $\beta$ -cytoplasmic actin followed by a Sulfolink column coupled with the NH<sub>2</sub>-terminal decapeptide of  $\gamma$ -smooth muscle actin to remove the population of antibodies cross-reacting with these 2 isoforms. Specificity of the fractions was tested by Western blotting.

### Electrophoretic and Immunoblot Analysis

Anti- $\alpha$ -SKA1 specificity was determined by immunoblotting of the following whole tissue homogenates: rat striated muscle, rat myocardium, human blood platelets, rat aorta, and chicken gizzard (40  $\mu$ g/lane were loaded for Coomassie blue staining and 2  $\mu$ g/lane for immunoblotting). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 5% to 20% gradient polyacrylamide gels,<sup>18</sup> and the proteins were electroblotted to nitrocellulose according to Towbin et al.<sup>19</sup> Nitrocellulose membranes were incubated with anti- $\alpha$ -SKA1 diluted in Tris-buffered saline solution (TBS) containing 3% BSA and 0.1% Triton X-100 for 2 hours at room temperature. After 3 washes with TBS, a second incubation was performed with peroxidase-conjugated affinity purified goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at a dilution of 1:10 000 in TBS containing 0.1% BSA and 0.1% Triton X-100. Peroxidase activity was developed using the enhanced chemiluminescence Western blotting system (Amersham) according to the instructions of the manufacturer. Blots were scanned (Arcus II; Agfa), and the intensity of the bands was quantified by means of the ImageQuant Program (Image Quant Analysis, Molecular Dynamics).

### Sedimentation Assays and Fab Fragment Preparation

Purification of actin from rabbit skeletal muscle acetone powder was carried out according to Spudich and Watt.<sup>20</sup> The effect of anti- $\alpha$ -SKA1 on  $\alpha$ -skeletal actin polymerization was examined as described by Chaponnier et al.<sup>17</sup>

Fab fragments were prepared by a slight modification of the method of Coulter and Harris.<sup>21</sup> Whole immunoglobulins were separated from other serum proteins on a Protein A-Sepharose 4B column (Pharmacia). After dialysis against 20 mmol/L phosphate and 10 mmol/L EDTA (pH 7.0), cysteine-HCl was added at a final

concentration of 10 mmol/L, and whole immunoglobulins were processed with immobilized papain (Pierce, 0.25 mL gel/mg immunoglobulin) overnight at 37°C. Papain beads were then separated from the digestion mixture and the undigested immunoglobulins were removed by chromatography on a Protein A column leaving in the flow through fraction a mixture of Fab and Fc fragments. The Fab fraction was affinity purified as described above for anti- $\alpha$ -SKA1.

### Experimental Models of Hypertension

A ligature was placed between renal arteries below the superior mesenteric artery of 300 g male Wistar rats.<sup>22</sup> This procedure results in a rapid development of a renin-dependent hypertension.<sup>23</sup> Animals were anesthetized with CO<sub>2</sub> and killed by cervical dislocation 3 or 7 days after ligature. Hearts were collected and used for RNA and protein extraction and immunofluorescence staining (see below). The experiments were approved by the Ethical Committee of Geneva Medical faculty.

### RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNAs were isolated from muscle tissues by Tri-Reagent (Molecular Research Center), according to the manufacturer's instruction. Each RNA sample was quantified spectrophotometrically at 260 nm before RT-PCR. The following primers were selected at the extremities of the 3' untranslated region of  $\alpha$ -skeletal actin because this region is isotype specific<sup>24</sup>: the sense primer was based on the complete gene sequence number 2985 to 3008 5'-CTCTCTCTCCTCAGGACGACAATC-3' and the antisense number 3168 to 3191 5'-CAGAATGGCTGGCTTTAATGCTTC-3'. Two micrograms of total RNA were first reverse-transcribed in the presence of 5 mmol/L of random primers p(dN)<sub>6</sub> (Boehringer-Mannheim AG) using the conditions previously described by Andreutti et al.<sup>25</sup> PCR in the linear range of amplification was performed to allow comparative analysis of numerous cDNA samples. The optimal reaction conditions chosen for the  $\alpha$ -skeletal actin-specific primers were the following: 2  $\mu$ L of the cDNA mixture were added to a master mix containing 1.25 mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 9.0; 0.1% Triton X-100; 0.02 mmol/L of each dNTP; 1.25 nmol/L of both sense and reverse primers; and 1U of Taq-DNA Polymerase (Promega). The PCR cycling parameters consisted of 20 seconds denaturation at 95°C, 30 seconds annealing at 60°C, and 30 seconds extension at 72°C for 25 cycles. Parallel  $\beta$ 2-microglobulin amplification was performed (annealing temperature 58°C, 25 cycles) with the following primers: 5'-ATCTTTCTGGTGCTTGCTC-3' (sense) and 5'-AGTGTGAGCCAGGATGTAGT-3' (antisense) (amplified fragment of 243-bp length). In each RT run, negative controls included the use of sterile water in place of the RNA to control the purity of the reagents and the absence of genomic DNA contamination (no amplified products were detected in this procedure, thus confirming the purity of RNA preparation). PCR products were then analyzed by electrophoresis on 2.5% agarose gel; 1  $\mu$ g of pGEM DNA (Promega) was used as marker. The amount of amplified products was quantified for each sample from scanned Polaroid negatives using ImageQuant software. Each value was determined as the mean of 3 densitometric readings. The final amount of PCR was expressed as the ratio of  $\alpha$ -skeletal actin gene amplified to that of  $\beta$ 2-microglobulin to account for any differences in starting amounts of RNA.

### Indirect Immunofluorescence, Confocal Laser Scanning Microscopy, and Light Microscopy

Tissue samples were embedded in OCT 4583 (Miles Scientific) and frozen in precooled liquid isopentane. Three- $\mu$ m cryostat sections were fixed in acetone at -20°C for 5 minutes and air-dried for 2 hours at room temperature. Sections were stained with purified anti- $\alpha$ -SKA1 (1:5 dilution in PBS) alone or combined with monoclonal antibodies against either human desmin (Clone 33, Dako),

connexin 43 (clone CX1B1, Zymed Laboratories),  $\beta$ -myosin heavy chain (clone 169-II-A2, kindly provided by Dr A Moorman, University of Amsterdam, The Netherlands,<sup>26,27</sup>) or  $\alpha$ -smooth muscle actin.<sup>14</sup> Subsequently, tissues were incubated with tetrahydroamine isothiocyanate-conjugated anti-rabbit and fluorescein-labeled anti-mouse antibodies (both from Jackson ImmunoResearch Laboratories).

Tissue sections were mounted in buffered polyvinyl alcohol<sup>28</sup> and observed with a confocal laser scan fluorescence inverted microscope (model LSM 410, Carl Zeiss). Images were stored on optical disks (Sony Corp) and printed with a digital Fujifilm Pictography 4000 printer (Fujifilm).

For light microscopy, tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Four- $\mu$ m sections were stained with hematoxylin-eosin and Masson's trichrome or processed for immunohistochemistry using anti- $\alpha$ -SKA1 (1:10 dilution in TBS) or anti- $\alpha$ -smooth muscle actin (1:50 dilution in TBS). Immunoperoxidase staining was performed as previously described.<sup>29</sup> Sections were observed using a Zeiss Axiophot photomicroscope. Images were acquired with a high sensibility Photonic Coolview color camera (Zeiss), stored, and printed as described above. Images were subsequently analyzed using the software KS400 (Kontron System, Zeiss Vision). Using this software, any structure could be selected on the basis of the pixel intensity values in each color channel. To measure the intensity of immunostaining, the image was transformed in gray luminance values ranging from 0 (corresponding to black) to 255 (corresponding to white). Results were given as the percentage of pixel number corresponding to a value below 200 (values above 200 were considered as background; Bochaton-Piallat, personal communication, 1998).

### Statistical Analysis

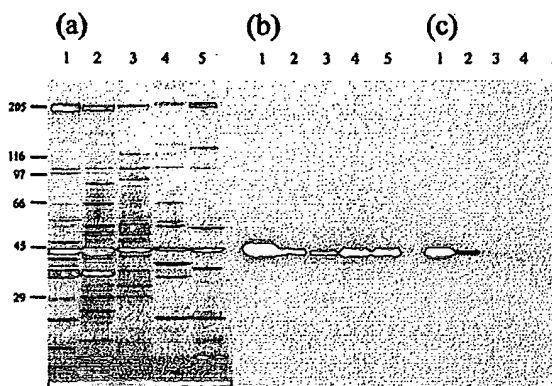
Results are shown as mean  $\pm$  SEM. For statistical comparison, the results were analyzed by the Student's *t* test.

## Results

### Anti- $\alpha$ -SKA1 Purification and Characterization

Immunization with hemocyanin-coupled  $\alpha$ -skeletal actin NH<sub>2</sub>-terminal decapeptide resulted in high titer antibodies showing an important cross-reactivity with all actin isoforms (data not shown). The first step of purification consisted in loading the immune serum on Sulfalink beads coupled with the decapeptide used for immunization. To test the cross-reactivity of the resulting antibodies with actin isoforms, we used extracts of rat striated muscle for  $\alpha$ -skeletal actin; rat myocardium for  $\alpha$ -cardiac actin; human blood platelets for  $\beta$  and  $\gamma$  cytoplasmic actins, known to be present at the ratio of 5 to 1<sup>30</sup>; rat aorta for  $\alpha$ -smooth muscle actin, representing 70% of total actin; and chicken gizzard for  $\gamma$ -smooth muscle actin.<sup>31</sup> Immunoblotting analysis (Figure 1b) revealed that the population of antibodies eluted from the beads coupled with the  $\alpha$ -skeletal decapeptide reacted with  $\alpha$ -skeletal actin but also with the other isoforms.

To remove antibody populations recognizing epitopes common to the 6 isoforms, we chose to first eliminate those reacting with the NH<sub>2</sub>-terminal decapeptide of  $\beta$ -cytoplasmic actin that shares the amino acid sequence ALV with the NH<sub>2</sub>-terminal decapeptide of  $\alpha$ -skeletal actin (see Figure 2B) and then to remove antibodies reacting with the NH<sub>2</sub>-terminal decapeptide of  $\gamma$ -smooth muscle actin. The resulting purified antibody population recognized the actin isoform present in the striated muscle extract but not actin from platelets, aorta, and gizzard (Figure 1c). The antibody also reacted with the cardiac muscle homogenate, albeit to a clearly lesser extent



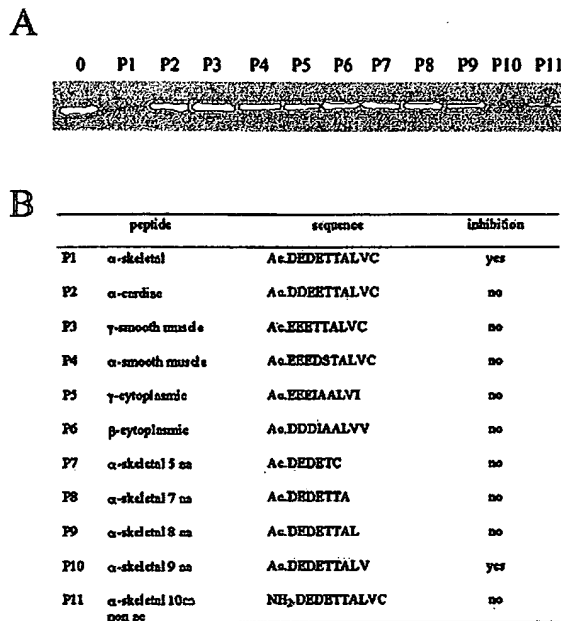
**Figure 1.** Characterization of anti- $\alpha$ -SKA1 specificity by Western blot. Specificity of anti- $\alpha$ -SKA1 was assessed at different steps of purification by immunoblots on different tissue or cell extracts. a, lanes 1 to 5 show Coomassie blue stained 5% to 20% gradient SDS-PAGE of rat skeletal muscle (lane 1), rat myocardium (lane 2), human blood platelets (lane 3), rat aorta (lane 4), and chicken gizzard (lane 5) (40  $\mu$ g/lane). b and c show corresponding nitrocellulose sheets immunostained with anti- $\alpha$ -SKA1 either after a first step of immunopurification on beads coupled with NH<sub>2</sub>-terminal decapeptide of  $\alpha$ -skeletal actin (b) or after the product of b was run on beads coupled with  $\beta$ -cytoplasmic and  $\gamma$ -smooth NH<sub>2</sub>-terminal decapeptides (c). In c, positive reaction is seen with skeletal muscle and, to a much lesser extent, cardiac extracts.

(Figure 1c, lane 2).  $\alpha$ -Skeletal actin has been shown to be coexpressed with  $\alpha$ -cardiac actin and to represent 5% of the sarcomeric actin mRNA in the normal adult rat cardiac tissue.<sup>5</sup> Thus, because all extracts were blotted after loading with similar amounts of actin (Figure 1a), it appeared likely that the reactivity of purified anti- $\alpha$ -SKA1 with the cardiac muscle extract is due to the fraction of myocardial  $\alpha$ -skeletal actin.

To further establish the specificity of purified anti- $\alpha$ -SKA1, we then investigated the amino acid residues involved in the interaction with the antibody by incubating anti- $\alpha$ -SKA1 with synthetic peptides corresponding to the NH<sub>2</sub>-terminal sequences of the 6 isoforms before immunoblotting analysis (Figure 2). The decapeptide of  $\alpha$ -skeletal actin used originally to raise the anti- $\alpha$ -SKA1 antiserum completely blocked the reactivity of purified anti- $\alpha$ -SKA1; however, peptides corresponding to the NH<sub>2</sub>-terminal sequence of the other vertebrate actins, including  $\alpha$ -cardiac actin, did not (Figure 2). Moreover, incubation of anti- $\alpha$ -SKA1 with the decapeptide of  $\alpha$ -skeletal actin inhibited the reactivity with myocardial extract (data not shown).

To identify the antibody epitope, a series of short peptides corresponding to the NH<sub>2</sub>-terminal sequence of  $\alpha$ -skeletal actin were synthesized (Figures 2A and 2B). Only the acetylated nonapeptide was able to fully compete with the antibody for actin. Thus the epitope recognized by the antibody includes the acetyl group and the first 9 amino acids of the  $\alpha$ -skeletal actin sequence.

Because an antibody against  $\alpha$ -smooth muscle actin recognizing the epitope Ac-EEED has been shown to specifically interfere with  $\alpha$ -smooth muscle actin in vitro polymerization,<sup>17</sup> the effect of anti- $\alpha$ -SKA1 on  $\alpha$ -skeletal actin



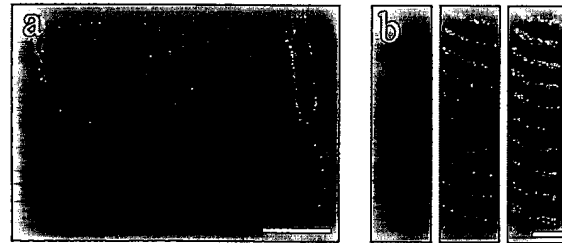
**Figure 2.** Blocking effect of NH<sub>2</sub>-terminal peptides on anti-α-SKA1 reactivity. A, Rat skeletal muscle homogenate (2 μg/lane) was subjected to SDS-PAGE in a 5% to 20% acrylamide gel followed by Western blotting with purified anti-α-SKA1 (1:25) alone (lane 0) or mixed with the different peptides (2 mg/mL diluted 1:10) listed in B (lanes P1 to P11). Only the NH<sub>2</sub>-terminal decapeptide (P1) and nonapeptide (P10) of α-skeletal actin blocked the immunoreactivity of the antibody.

polymerization was investigated. The protocol followed for this purpose has been previously described.<sup>17</sup> Briefly, we compared the amount of actin present in the pellets and supernatants after sedimentation assays performed either in physiological salt conditions (100 mmol/L KCl, 2 mmol/L MgCl<sub>2</sub>) or in the presence of anti-α-SKA1 (1 μmol/L final concentration) in the same salt conditions. In salt conditions, 25% of α-skeletal actin partitioned in the insoluble fraction. α-Skeletal actin polymerization was significantly enhanced when anti-α-SKA1 was included in the incubation mixture (with more than 70% of the protein recovered in the pellet, data not shown). However, the anti-α-SKA1 Fab, with only 1 actin-binding site, had no significant effect on actin polymerization.

The specificity of purified anti-α-SKA1 was then tested by means of immunofluorescence on adult rat stretched<sup>32</sup> skeletal muscle. As shown in Figure 3, the only positive staining was located in muscle fibers with the classical banding pattern. Confocal microscopy demonstrated that, as expected, desmin and anti-α-SKA1 stainings are located in the Z- and I-bands of myocytes, respectively (Figure 3b).

#### Immunolocalization of α-Skeletal Actin in Myocardium

Anti-α-SKA1 immunostaining was again localized in myocardial fibers with a banding pattern. Unexpectedly, however, α-skeletal actin was restricted to a subpopulation of ventricular cardiomyocytes randomly distributed throughout the



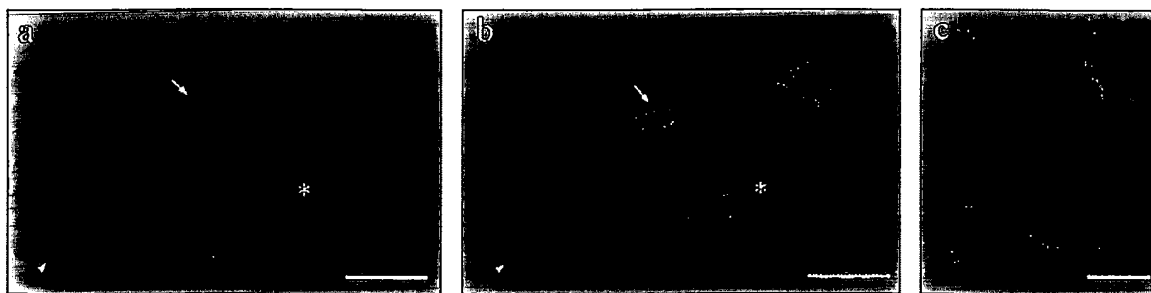
**Figure 3.** Characterization of anti-α-SKA1 by double immunofluorescence. Adult rat skeletal muscle sections were double-labeled by indirect immunofluorescence with purified anti-α-SKA1 (red in a and b) and monoclonal antibodies against either α-smooth actin (green in a) or desmin (green in b). Tissues were then observed with a confocal laser scan fluorescence microscope. Anti-α-SKA1 does not stain vascular smooth muscle but is restricted to muscle fibers (a). In b, anti-desmin and anti-α-SKA1 stain Z-lines and I-bands, respectively. Scale bars=100 μm (a) and 5 μm (b).

sections (Figure 4a). Double staining with anti-α-SKA1 and anti-connexin 43, the principal gap junction protein located in intercalated disks further confirmed the specific localization of α-skeletal actin in distinct cardiomyocytes (Figure 4c). Staining intensity was variable in different cells, suggesting a spectrum of α-skeletal actin expression. Positively-stained cells were consistently more abundant in the left compared with the right ventricle and staining was not detectable in the atria (data not shown).

A similar pattern of focal staining has been described for cardiac myosin isoforms by immunochemical studies as well.<sup>33–35</sup> To investigate a possible colocalization of α-skeletal actin and β-myosin heavy chain, we performed double staining with anti-α-SKA1 and an antibody specific for β-myosin heavy chain.<sup>26,27</sup> An important degree of coexpression was observed (56.0±4.1% of β-myosin heavy chain positive cells expressed also α-skeletal actin); however, cardiomyocytes expressing only 1 of the 2 proteins were also present (Figures 4a and 4b).

#### Evaluation of α-Skeletal Actin mRNA and Protein Content and Distribution in Normal Newborn and Adult, and in Hypertensive Rat Myocardium

α-Skeletal actin mRNA is known to represent up to 40% of the total sarcomeric actin mRNA in newborn rats and then to decrease to <5% at 2 months of age.<sup>5</sup> The availability of anti-α-SKA1 allowed us to investigate the modulation of α-skeletal actin during postnatal development at the protein level by means of immunoperoxidase staining and Western blot analysis (Figure 5). Unlike what is observed in normal adult cardiac muscle (Figure 5A, panel b), the myocardium of 3-day-old rats displayed a uniform pattern of reactivity (Figure 5A, panel a). Thus focal distribution of α-skeletal actin in myocardial fibers takes place during development. Western blot analysis confirmed that α-skeletal actin content is 4-fold higher in newborn compared with adult heart (Figure 5B). Analysis of RNA expression by RT-PCR corroborated the results obtained for the protein in that the ratio of PCR product of α-skeletal actin mRNA/β2-microglobulin mRNA obtained for newborn sample also was approximately 4-fold greater than that of normal adult (Figure 5C).



**Figure 4.** Double-labeling indirect immunofluorescence of normal rat cardiac muscle frozen sections. Tissue was stained with purified anti- $\alpha$ -SKA1 (red in a and c) and a monoclonal antibody directed against either anti- $\beta$ -myosin heavy chain (green in b) or connexin 43 (green in c). Anti- $\alpha$ -SKA1 stained a subpopulation of fibers in normal rat cardiac muscle. Intercalated disks were clearly defined by connexin antibody; double staining showed that  $\alpha$ -skeletal actin was limited to specific fibers (c). A significant proportion of anti- $\alpha$ -SKA1 positive myocardial fibers was also positive for  $\beta$ -myosin heavy chain (a and b, asterisks). However, some cardiomyocytes highly positive for  $\alpha$ -skeletal actin exhibited no reactivity for  $\beta$ -myosin heavy chain (arrowhead) and vice versa (arrow). Scale bars=50  $\mu$ m (a and b) and 25  $\mu$ m (c).

Acute hypertension induced by aortic ligation between renal arteries resulted in cardiac hypertrophy (ventricular wet weight/body weight  $4.48 \pm 0.61$  mg/g compared with  $2.47 \pm 0.09$  in control rats  $P < 0.001$  and see also Reference 34). Immunohistochemical staining with anti- $\alpha$ -SKA1 revealed that the number of positive myocardial fibers was significantly increased 3 and 7 days after aorta ligation (Figure 5A, panel c) compared with the number of positive fibers present in normal heart. The area of positive staining increased from  $4.2 \pm 0.5\%$  in the normal heart to  $31.5 \pm 2.4\%$  in the hypertensive heart 7 days after aortic ligation ( $P < 0.001$ ). Reactivity to anti- $\alpha$ -SKA1 also occurred in left atrium, where it was undetectable in the normal situation (data not shown). At the same time, reactivity remained negative in the right atrium of hypertensive animals (data not shown). The increase of protein expression was also confirmed by means of immunoblotting (Figure 5B, 4-fold higher compared with normal adult) and at the mRNA level (Figure 5C, 2-fold increase of the signal). The expression of  $\beta$ -myosin heavy chain was increased during hypertension; however, in contrast to what was observed for anti- $\alpha$ -SKA1, the staining pattern for  $\beta$ -myosin became diffuse throughout the ventricular myocardium confirming the findings of Gorza et al.<sup>34</sup> (data not shown).

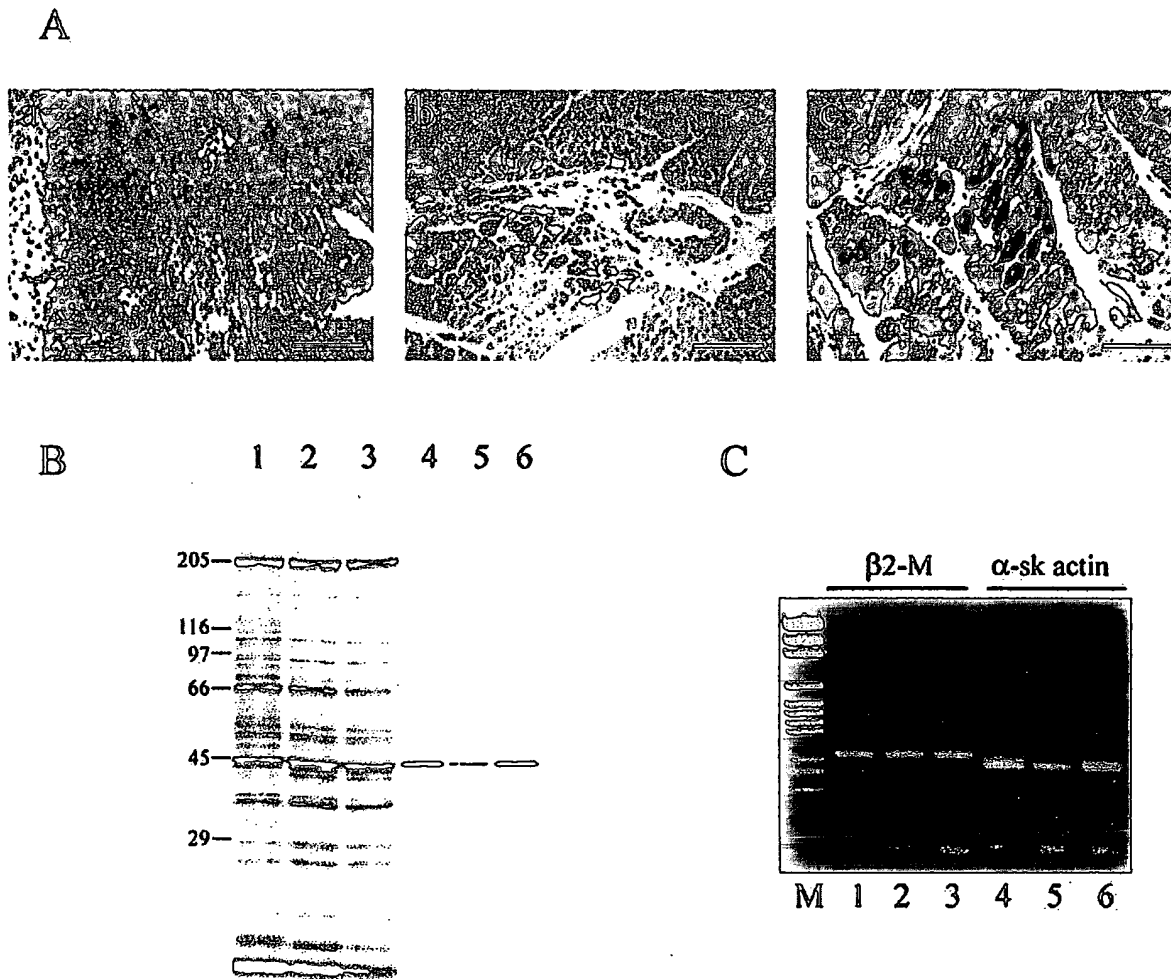
Spots of fibrosis were visible 7 days after the beginning of the experiment on examination of Masson's trichrome-stained sections (Figure 6a). These fibrotic areas were characterized by the expression of  $\alpha$ -smooth muscle actin, the actin isoform typical of myofibroblastic cells (Figure 6b), which are known to be abundant and persistent in granulation tissue developing after cardiac infarction.<sup>36,37</sup> Interestingly, fibers surrounding the fibrotic areas in the hypertensive animals consistently demonstrated an important staining for  $\alpha$ -skeletal actin (Figure 6c).

### Discussion

Previous attempts to develop antibodies specific for  $\alpha$ -skeletal or  $\alpha$ -cardiac actin isoforms have not so far allowed to discriminate between these isoforms,<sup>38-41</sup> with the exception of the anti- $\alpha$ -cardiac actin antibody raised by Franke et al.,<sup>16</sup> the specificity of which is dependent of the experimental

conditions. This is not unexpected because the divergences among the 2 sequences are minimal. The experimental strategy we used here shows that it is possible to produce in the rabbit and immunopurify an antibody that reacts specifically with  $\alpha$ -skeletal actin. This suggests the possibility of obtaining monospecific antibodies for other actin isoforms insofar as they are not presently available because the sequences of  $\alpha$ -cardiac and  $\alpha$ -skeletal actins are the most difficult to discriminate. The fractions obtained after the first step of immunopurification (on beads coupled with the  $\alpha$ -skeletal actin decapeptide used for immunization) resulted in several antibody populations, indicating that the decapeptide contains several epitopes. The further and successful purification we used was based on the removal of antibody populations recognizing the sequence ETTALVC (positions 4 to 10) common to  $\alpha$ -skeletal,  $\alpha$ -cardiac, and  $\gamma$ -smooth muscle actins, and the resulting antibody showed a high specificity for  $\alpha$ -skeletal actin. These results are compatible with the assumption that the Ac-DED sequence represents a good candidate for the  $\alpha$ -skeletal actin-specific epitopic activity, as it corresponds to the only difference between the NH<sub>2</sub>-terminal end of  $\alpha$ -skeletal actin and that of  $\gamma$ -smooth muscle actin (Ac-EEE) (see Figure 2B). However, inhibition experiments with synthetic peptides show that the first 9 amino acids are essential for antibody recognition. Possibly, a particular 3D conformation is involved in this process. The blocking acetyl group appears to be an integral part of the epitope because the nonacetylated decapeptide does not block anti- $\alpha$ -SKA1 reactivity. Incubation of the Fab fragment of anti- $\alpha$ -SKA1 does not influence  $\alpha$ -skeletal actin polymerization, differently to what we have previously reported for the corresponding fragment of  $\alpha$ -smooth muscle actin antibody,<sup>17</sup> indicating that for polymerization, the epitopic region of this antibody is not as important as that of  $\alpha$ -smooth muscle actin reacting with the specific antibody.

To date, it is not clear whether the 2 striated muscle actins have different physiological properties. Studies with probes for mRNAs, the only tools available so far to distinguish between  $\alpha$ -skeletal and  $\alpha$ -cardiac actin isoforms, have shown that both sarcomeric actin transcripts are expressed in skeletal and cardiac muscles and that their expression is tightly



**Figure 5.**  $\alpha$ -Skeletal actin distribution in normal newborn and adult, and in hypertensive rat myocardium. **A**, Immunoperoxidase staining with anti- $\alpha$ -SKA1 on formal fixed normal neonatal (a), adult (b), and in hypertensive (c) myocardium. Note the decrease in extension and the change in distribution of  $\alpha$ -skeletal actin positive staining occurring in myocardium between newborn and adult stages (a compared with b). However, the distribution remains focal as it is in the normal adult myocardium. Scale bars=100  $\mu$ m. **B**, Cardiac muscle homogenates of 3-day-old neonatal (lanes 1 and 4), normal adult (lanes 2 and 5), and hypertensive (lanes 3 and 6) rats were analyzed by SDS-PAGE on a 5% to 20% acrylamide gel stained with Coomassie blue (lanes 1 to 3) and immunoblotted with anti- $\alpha$ -SKA1 (lanes 4 to 6). The levels of  $\alpha$ -skeletal actin expression clearly decrease in the adult rat (lane 5) compared with the newborn (lane 4) and increase again in the hypertensive rat (lane 6). **C**, Detection of  $\alpha$ -skeletal actin transcripts by RT-PCR. A representative set of bands of  $\alpha$ -skeletal actin transcripts in newborn (lane 4), normal adult (lane 5), and hypertensive (lane 6) myocardium is presented. RT-PCR products for  $\beta$ 2-microglobulin ( $\beta$ 2-M) are included as control to normalize the starting amount of RNA (lane 1, newborn; lane 2, normal adult; lane 3, hypertensive heart). Lane M contains a DNA-size marker. The levels of  $\alpha$ -skeletal actin message correlate with the protein expression shown in A and B.

regulated during development.<sup>5</sup> These results provide a strong argument in favor of functional differences between these isoforms. Further investigations on their cellular distribution should lead to a greater insight into their respective biological functions during muscle development or pathology. As a first approach to such a study, *in situ* hybridization procedures performed by Schiaffino et al<sup>11</sup> did not detect  $\alpha$ -skeletal actin mRNA labeling in normal adult rat heart; this labeling, however, was important and was uniformly distributed throughout the ventricle soon after aortic ligation. Our investigations at the protein level confirm the increased expression

of this isoform during cardiac hypertrophy. However, we show that  $\alpha$ -skeletal actin protein is focally present in normal adult myocardial fibers. The reasons for this apparent discrepancy between *in situ* hybridization and immunolabeling are presently not clear.

Our results indicate that there may be a relationship between the expression of  $\alpha$ -skeletal actin and  $\beta$ -myosin. Thus a high percentage of myocytes expresses both proteins, although this is not uniformly the case. Furthermore, during the development of hypertrophy, the distribution of  $\alpha$ -skeletal actin remains focal, whereas it is known that the distribution



**Figure 6.**  $\alpha$ -Skeletal actin expression in myocardial cells close to a fibrotic area in a hypertensive rat. Adjacent sections were stained with Masson's trichrome (a), anti- $\alpha$ -smooth muscle actin (b), and anti- $\alpha$ -SKA1 (c).  $\alpha$ -Skeletal actin is strongly expressed in cardiomyocytes surrounding the fibrotic area which is characterized by the presence of collagen (a, blue staining) and the expression of  $\alpha$ -smooth muscle actin (b, brown staining). Scale bar=200  $\mu$ m.

of  $\beta$ -myosin is more diffuse.<sup>34</sup> Further work along this line may help in understanding the physiological role of both proteins.

Several studies have demonstrated that there is increased expression of  $\alpha$ -skeletal actin during the evolution of cardiac hypertrophy.<sup>3,4,42</sup> One of the explanations that has been offered is that elements of the genetic programming necessary during development may be reactivated, which could result in the re-expression of fetal genes. Our investigations of postnatal development show that the distribution of  $\alpha$ -skeletal actin is uniform within the myocardium of newborn rats and becomes focal as development proceeds. During hypertension-induced hypertrophy, however, the pattern of  $\alpha$ -skeletal actin reactivity does not become diffuse but rather remains focal. This suggests that the mechanisms of  $\alpha$ -skeletal actin gene regulation during development are different from those occurring during the evolution of hypertrophy. It has also been proposed that the activation of the  $\alpha$ -skeletal actin gene during the development of hypertrophy reflects a temporary requirement for large quantities of striated muscle actin when muscle volume is increasing rapidly.<sup>1</sup> The particular pattern of  $\alpha$ -skeletal actin distribution suggests that the expression of this actin isoform is associated with specialized functions of certain cardiomyocytes. Taken together with our observation that  $\alpha$ -skeletal actin-positive cells are more abundant in the left compared with the right ventricle and that their number increases during hypertrophy, our results are in accordance with the hypothesis that this actin isoform may be required to achieve a higher degree of myocardial contractility as indicated by the findings of Hewett et al.,<sup>43</sup> showing that increased levels of  $\alpha$ -skeletal actin mRNA are significantly correlated with increased heart contractility in BALB/c mice. The observation that fibers close to fibrotic areas express high amounts of  $\alpha$ -skeletal actin during hypertrophy are compatible with the possibility that these particular fibers are submitted to stronger local physical forces and/or to particular cytokines or growth factors liberated in the fibrotic areas. In this respect it is noteworthy that locally produced transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) plays an important role in the establishment of fibrotic changes (for review see Reference 44); TGF- $\beta$ 1 has also been shown to induce a selective upregulation of the  $\alpha$ -skeletal actin promoter.<sup>45-47</sup>

In conclusion, our success in producing a specific  $\alpha$ -skeletal actin antibody suggests that the strategy used in the present work may be attempted for other actin isoforms. Our results indicate that during development, there is a modula-

tion of  $\alpha$ -skeletal actin expression such that uniform distribution in the newborn gives way to a focal pattern in the adult. The pattern of expression during development of hypertrophy also is focal; moreover, the reactivity to anti- $\alpha$ -SKA1 is preferentially associated with regions of fibrosis. Further studies with this antibody will be useful in the understanding of the relationship between  $\alpha$ -skeletal actin expression and striated muscle function.

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